DESCRIPTION

ANTI-HIV ANTIBODY

TECHNICAL FIELD

The present invention relates to an antibody that binds to gp120 (a glycoprotein with a molecular weight of about 120 kD in the envelope of HIV) with high affinity, and a cell producing the same. The present invention also relates to a pharmaceutical composition comprising the above antibody.

BACKGROUND ART

Acquired immunodeficiency syndrome (AIDS) refers to a condition of HIV (human immunodeficiency virus) infected individuals where immunopotency has gradually decreased and complications easily occur.

Once HIV invaded the body of a host, HIV infects CD4 positive cells, particularly $CD4^+$ T lymphocytes (helper T cells). The protein that is involved in HIV infection of CD4 positive cells is an HIV envelope protein called gp120, which is a glycoprotein with a molecular weight of about 120 kD present in the envelope of HIV and binds to CD4 molecule on cell surfaces as a specific receptor. After infecting $CD4^+$ lymphocytes, HIV invades inside of cells, undergoes uncoating and liberates its nucleic acid (RNA). Then, DNA is synthesized by reverse transcriptase, transcribed and translated. Thus, viral proteins are synthesized. The viral proteins migrate to cell membranes to form virions, which are then released.

Since the antigenic drift of HIV is very frequent, vaccine preparation is difficult and no effective vaccine has been developed yet. Further, since HIV gene is integrated into the chromosomes of infected cells, it is extremely difficult to conduct a drastic treatment of removing the infecting HIV completely.

Currently, drugs such as AZT (azidothymidine) are recognized to be effective in postponing the onset of AIDS and prolonging the lives of patients; and new therapeutics with promising efficacy are being developed one by one. However, no decisive therapeutic has been established yet.

On the other hand, various attempts have been made to obtain antibodies that have the ability to effectively neutralize HIV and are useful for prevention or diagnosis of AIDS. Since gp120 is one of the most important molecules for HIV infection (McDougal et al.,

Science, 231,382-385 (1986)), it is possible to target gp120 in effective inhibition, prevention and diagnosis of HIV infection. An antibody called "0.5β" has already been prepared which recognizes one epitope present within amino acids 308-331 of the amino acid sequence of gp160, a precursor of the HIV gp120 (Japanese Patent No. 2797099). However, for further enhancing the avidity to antigen, it is necessary to develop high affinity antibodies that can react with the HIV gp120 and effectively neutralize the virus.

DISCLOSURE OF THE INVENTION

It is an object of the present invention to provide a high affinity antibody having the ability to neutralize HIV and a pharmaceutical composition comprising the antibody. It is another object of the invention to provide a pharmaceutical composition useful in treating acquired immunodeficiency syndrome.

As a result of intensive and extensive researches toward the solution of the above problems, the present inventors have found that a GANP transgenic non-human mammal immunized with gp120 produces an antibody that neutralizes the activity of HIV and binds to HIV with high affinity. Thus, the present invention has been achieved.

The present invention relates to the following.

(1) An antibody or a fragment thereof which binds to the gp120 glycoprotein of HIV and has a dissociation constant (KD) value of 1.0×10^{-9} (M) or less.

The above-described antibody or fragment thereof is capable of recognizing at least a part of an amino acid sequence spanning from amino acid positions 308 to 330 of the gp120 glycoprotein (e.g., the amino acid sequence as shown in SEQ ID NO: 6).

The antibody or fragment thereof of the present invention may be an antibody collected from a serum of a non-human mammal; the antibody may be a polyclonal antibody or a monoclonal antibody.

The antibody or fragment thereof of the present invention is produced, for example, by a hybridoma cell having an accession number of FERM BP-08644 [designation: "Anti-NL43mono. Clone No. G2-25 hybridoma cell"; depository: International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology (Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan; zip code No. 305-8566); date of deposit: February 25, 2004].

(2) A humanized antibody or human antibody, or a fragment thereof, which comprises the V region of the above-described antibody or fragment thereof.

(3) A high affinity antibody-producing cell collectable from a GANP transgenic

non-human mammal, or a progeny thereof, that was immunized with a polypeptide comprising at least a part of the amino acid sequence as shown in SEQ ID NO: 6 as an antigen.

The present invention also provides a cell producing a monoclonal antibody to the gp120 glycoprotein of HIV, which has an accession number of FERM BP-08644.

(4) A method of producing an anti-HIV antibody or a fragment thereof, comprising immunizing a GANP transgenic non-human mammal or a progeny thereof with a polypeptide comprising at least a part of the amino acid sequence as shown in SEQ ID NO: 6 as an antigen and collecting the antibody from the resultant mammal or progeny.

(5) A method of producing an anti-HIV antibody or a fragment thereof, comprising culturing a fusion cell composed of the high affinity antibody-producing cell of (3) above and a myeloma cell, or the monoclonal antibody-producing cell having an accession number of FERM BP-08644, and collecting the antibody from the resultant culture.

(6) A pharmaceutical composition comprising at least one selected from the group consisting of the antibody or fragment thereof of (1) above and the humanized antibody or human antibody, or fragment thereof of (2) above.

The pharmaceutical composition of the present invention may be used as a therapeutic for acquired immunodeficiency syndrome.

(7) A method of detecting HIV, comprising reacting the antibody or fragment thereof of (1) above, or the humanized antibody or human antibody, or fragment thereof of (2) above with the gp120 glycoprotein of HIV.

(8) An HIV detection kit comprising at least one selected from the group consisting of the antibody or fragment thereof of (1) above and the humanized antibody or human antibody, or fragment thereof of (2) above.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is panels showing increases of GANP expression in B cells.

Fig. 2 is a graph showing the results of detection of gp120(308-330) peptide with individual antibodies by ELISA.

Fig. 3 is a graph showing the results of measurement of dissociation constant in individual clones.

Fig. 4 is a graph showing the results of evaluation of the binding abilities of individual anti-HIV monoclonal antibodies to HIV envelope.

Fig. 5 is a graph showing the results of evaluation of the binding abilities of individual anti-HIV monoclonal antibodies to HIV envelope.

Fig. 6 is a graph showing the results of neutralizing activity test on individual anti-HIV monoclonal antibodies.

Fig. 7 is a graph showing the results of neutralizing activity test on individual anti-HIV monoclonal antibodies.

BEST MODE FOR CARRYING OUT THE INVENTION

1. Outline

The antibody of the invention is obtainable by immunizing a GANP transgenic mammal with a part of the gp120 of HIV, in particular, a peptide consisting of an amino acid sequence spanning from amino acid positions 308 to 330 in the amino acid sequence of gp120 as an antigen. Although it is known that an antibody recognizing the partial amino acid sequence spanning from amino acid positions 308 to 330 in the amino acid sequence of gp120 (hereinafter, referred to as "gp120(308-330)") has virus neutralizing activity and inhibitory activity against the syncytium formation by infected cells (Skinner MA. et al., AIDS Res. Hum. Retroviruses (1988), 4(3), 187-197), the antibody of the present invention is characterized by binding to gp120(308-330) with high affinity.

GANP is a nuclear protein called germinal center-associated nuclear protein. GANP is a molecule needed directly or indirectly in the process of induction of mutations in genes. Since GANP has the ability to promote induction of mutations in the V region so that high affinity antibodies are obtained. Therefore, a transgenic non-human mammal into which a gene encoding GANP has been introduced (hereinafter, called the "GANP transgenic non-human mammal") is, as a result of the GANP gene introduction, capable of promoting the production of high affinity antibodies of acquired immunity. Further, this GANP transgenic non-human mammal is capable of promptly producing antibodies with high avidity to antigens. Therefore, by immunizing the above-described GANP transgenic non-human mammal with a peptide consisting of a partial amino acid sequence of HIV gp120 (for example, gp120 (308-330)) as an antigen, it is possible to obtain easily antibodies with high affinity which were unobtainable by conventional methods.

As described so far, according to the present invention, it becomes possible to obtain anti-HIV antibodies having HIV neutralizing activity, inhibitory activity against syncytium formation by infected cells, and high affinity that could not be achieved by conventional methods. Further, a pharmaceutical composition comprising the resultant antibody may be used for treating AIDS.

Cells producing the above-described antibody may be splenic B cells or lymph node cells alone obtained from a GANP transgenic non-human mammal immunized with

gp120. Alternatively, the antibody-producing cell may be a hybridoma cell obtained by fusing such B cells or lymphocytes with a myeloma cell. The present invention also provides a cell producing the above-described antibody.

In clinical tests conducted to confirm HIV infection, it is important to detect HIV with high sensitivity. The high affinity anti-HIV antibody of the invention may be used as a means to detect HIV. Therefore, the present invention provides an HIV detection kit comprising the anti-HIV antibody of the invention.

2. Preparation of Antigen

Sequence information for HIV gp120 may be obtained from databases or the like (PRF 1102247A, http://www.genome.ad.jp/dbget-bin/www_bget?prf:1102247A); the amino acid sequence of gp120 is as shown in SEQ ID NO: 5.

The polypeptide sequence of gp120(308-330) consists of the 23 amino acid residues as described below (Lee Ratner et al., Nature 313, 277-284, 1985):

NNTRKSIRIQRGPGRAFVTIGKI (SEQ ID NO: 6)

A polypeptide or peptide (sometimes simply referred to as "peptide") comprising at least a part (i.e., the whole or a part) of the amino acid sequence may be used as an antigen.

It should be noted here that "at least a part of the amino acid sequence" of the peptide sequence represented by SEQ ID NO: 6 to be used as an antigen is not particularly limited in length. For example, 8 or more consecutive amino acid residues in the 23 amino acid residues (e.g., 8, 10, 12, 16, 20 or 23 amino acid residues) may be included. Any site may be selected as long as the site consists of consecutive amino acids in SEQ ID NO: 6. For example, when 7 to 8 amino acids are to be used as an antigen, the amino acid sequences of three regions each consisting of 7 to 8 amino acids may be selected from the N-terminus toward the C-terminus of the amino acid sequence consisting of the 23 amino acid residues shown in SEQ ID NO:6. Alternatively, the amino acid sequence of a region consisting of 7 to 8 amino acids starting from the N-terminus may be selected, and then the amino acid sequence of subsequent regions serially shifted toward the C-terminus by one amino acid at one time may be selected in succession.

SEQ ID NO: 6 and the above-described at least a part of this amino acid sequence may be used independently or in combination, as an antigen.

Alternatively, the above-described peptide may be linked to a carrier protein to prepare an antigen that has a large number of the peptide as side chains. In this case, it is possible to add a cysteine residue to the N-terminus to the above peptide in order to allow a carrier protein to bind thereto.

The peptide may be prepared by chemical synthesis or biochemical synthesis using *Escherichia coli* or the like. Methods well-known in those skilled in the art may be used for the synthesis.

When the peptide of the invention is chemically synthesized, methods well-known in the field of peptide synthesis may be used. For example, such methods as the azide method, the acid chloride method, the acid anhydride method, the mixed acid anhydride method, the DCC method, the active ester method, the carboimidazole method and the oxidation-reduction method may be enumerated. Either solid phase synthesis or liquid phase synthesis may be used. A commercial peptide synthesizer (e.g., Shimadzu PSSM-8) may also be used.

After the reaction, the peptide of the invention may be purified by a combination of conventional purification methods such as solvent extraction, distillation, column chromatography, liquid chromatography or re-crystallization.

When the peptide of the invention is biochemically synthesized, a DNA encoding the peptide is designed and synthesized. Then, this DNA is ligated to an appropriate vector to thereby obtain a recombinant vector for protein expression. By introducing this recombinant vector into a host in such a manner that the gene of interest is expressed, a transformant can be obtained (Sambrook J and Russel D., Molecular Cloning, A Laboratory Manual, 3rd edition, CSHL Press, 2001).

As a vector, a phage or plasmid capable of autonomous replication in a host microorganism is used. Examples of plasmid DNA include plasmids derived from *Escherichia coli*, *Bacillus subtilis* or yeast, and examples of phage DNA include λ phage. An animal virus vector or insect virus vector may also be used.

A recombinant vector may be prepared by digesting a purified DNA with appropriate restriction enzymes and inserting the digest into an appropriate restriction site or the like of a vector DNA for ligation.

The host used for transformation is not particularly limited as long as it is capable to expressing the gene of interest. Examples of hosts include bacteria (*Escherichia coli*, *Bacillus subtilis*, etc.), yeast, animal cells (COS cells, CHO cells, etc.), incest cells or insects. It is also possible to use a mammal such as goat as a host.

Methods of introduction of recombinant vectors into hosts are known. Any method (such as the method using calcium ions, electroporation, the spheroplast method, the lithium acetate method, the calcium phosphate method or lipofection) may be included in the methods.

In the present invention, the peptide of the invention may be obtained by culturing

the above-described transformant and collecting from the resultant culture. The term "culture" used herein means any of the following materials: (a) culture supernatant, (b) cultured cells or cultured microorganisms, or a disrupted product obtained therefrom.

Culture method is well-known in the art (see Sambrook et al., Molecular Cloning, *op. cit.*).

After culturing, when the peptide of interest is produced in the microorganism or cell, the microorganism or cell is disrupted to extract the peptide. When the peptide of interest is produced outside the microorganism or cell, the culture broth may be used as it is or centrifuged to remove the microorganism or cell. Subsequently, the peptide of interest may be isolated/purified using common biochemical methods used in peptide isolation/purification (such as ammonium sulfate precipitation, gel filtration, ion exchange chromatography or affinity chromatography) independently or in combination.

In the present invention, it is also possible to adopt peptide synthesis by *in vitro* translation. Two methods may be applicable to this synthesis; one is a method using RNA as a template and the other is a method using DNA as a template (transcription/translation). As a template DNA, a DNA encoding the above peptide having a promoter and a ribosome binding site upstream of the translation start point or a DNA in which necessary elements for transcription (e.g., promoter) are integrated upstream of the translation start point may be included. As an *in vitro* translation system, a commercial system such as Expressway™ system (Invitrogen), PURESYSTEM (registered trademark; Post Genome Institute) or TNT system (registered trademark; Promega) may be used. After synthesis by an *in vitro* translation system, the peptide of interest can be isolated/purified by using the above-described common biochemical methods independently or in combination.

As a carrier protein to be linked to the thus obtained peptide, bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), human thyroglobulin or chicken gamma globulin may be enumerated.

3. GANP

GANP is a 210 kD nuclear protein having homology to yeast Sac3 protein (WO 00/50611). SAC3 is characterized as an inhibitory substance against actin formation. It is known that GANP is selectively up-regulated in germinal center (GC) B cells surrounded by follicular dendritic cells: FDC), has phosphorylation-dependent RNA primase activity, and is involved in the regulation of the cell cycle of B cells (Kuwahara, K. et al., (2000) Blood 95: 2321-2328).

In the present invention, the amino acid sequence for mouse GANP protein is

shown in SEQ ID NO: 2 and the amino acid sequence for human GANP protein is shown in SEQ ID NO: 4. With respect to the gene encoding the GANP protein (hereinafter, referred to as "GANP gene"), the nucleotide sequence for mouse GANP gene is shown in SEQ ID NO: 1 and the nucleotide sequence for human GANP gene is shown in SEQ ID NO: 3. The above-mentioned amino acid sequences and nucleotide sequences are also described in WO 00/50611.

GANP proteins may be mutant proteins; they may be those proteins which consist of the amino acid sequence as shown in SEQ ID NO: 2 or 4 wherein one or a plurality of amino acids have been deleted, substituted or added and have RNA primase activity. For example, a GANP mutant protein may also be used which consists of the amino acid sequence as shown in SEQ ID NO: 2 or 4 wherein one or a plurality of amino acids (preferably, one or several (e.g. one to ten, more preferably one to five) amino acids) have been deleted, one or a plurality of amino acids (preferably, one or several (e.g. one to ten, more preferably one to five) amino acids) have been substituted with other amino acids, and/or one or a plurality of other amino acids (preferably, one or several (e.g. one to ten, more preferably one to five) amino acids) have been added thereto, and yet has the same RNA primase activity as that of the above-described GANP protein.

"RNA primase activity" means the enzyme activity synthesizing a short primer RNA which will be a starting point for strand elongation when a strand extending opposite to the 5' → 3' direction (lagging strand) is synthesized in RNA replication. Usually, a molecule called α primase, which binds to DNA polymerase α is used. In germinal center B cells, GANP primase which is the second primase is also induced.

GANP protein includes a protein having the amino acid sequence as shown in SEQ ID NO: 2 or 4, or a mutant amino acid sequence thereof, and a protein having a part of the N-terminal sequence of those sequences (e.g. positions from 1 to 600, preferably from 139 to 566 of the amino acid sequence as shown in SEQ ID NO: 2) or a mutant amino acid sequence thereof.

In the present invention, a GANP gene to be transferred into an animal may be a gene encoding the above-described GANP protein, a part of the N-terminal sequence of the GANP protein, or a mutant GANP protein. Specific examples of such a gene include a gene having the nucleotide sequence as shown in SEQ ID NO: 1 or 3. A gene having only the coding region of the nucleotide sequence as shown in SEQ ID NO: 1 or 3 may also be used. Alternatively, it is also possible to use a gene that has a sequence hybridizable to a complementary sequence to the nucleotide sequence as shown in SEQ ID NO: 1 or 3 under stringent conditions, and encodes a protein having RNA primase activity.

"Stringent conditions" refers to washing conditions after hybridization; specifically, the salt (sodium) concentration is 150-900 mM and the temperature is 55-75°C, preferably salt (sodium) concentration is 250-450 mM and the temperature is 68°C.

Introduction of mutations into a gene may be performed according to known techniques such as the Kunkel method or the gapped duplex method, using mutagenesis kits utilizing site-directed mutagenesis, such as GeneTailor™ Site-Directed Mutagenesis System (Invitrogen) or TaKaRa Site-Directed Mutagenesis System (Mutan-K, Mutan-Super Express Km, etc.; Takara Bio), etc.

Details of mutant genes and methods for obtaining the same are also described in WO 00/50611.

In vitro stimulation of B cells with anti-μ antibody and anti-CD40 monoclonal antibody induces not only the up-regulation of GANP expression but also the phosphorylation of a specific serine residue in the amino acid sequence of GANP protein (e.g. serine at position 502: S502). This reaction is a key reaction for the RNA primase activity of GANP (Kuwahara, K. et al. (2001) Proc. Natl. Acad. Sci. USA, 98, 10279-10283). The N-terminal primase domain of GANP protein contains a serine residue whose phosphorylation is catalyzed by Cdk2 *in vitro*. GANP binds to MCM3 replication licensing factor due to its C-terminal domain (Kuwahara, K. et al., (2000) Blood 95: 2321-2328; Abe, E. et al., (2000) Gene 255: 219-227).

GANP gene-deficient mice are embryonic lethal. The inventors created a conditional targeting mouse selectively lacking GANP gene in B cell by crossing CD19-Cre mouse with flox-ganp mouse, immunizing the resultant mouse with nitrophenyl (NP)-chicken gamma globulin antigen (T cell dependent antigen) and examined the mouse for NP-hapten specific antibody production. The results revealed that the high affinity antibody production in this mouse is remarkably hindered, indicating that GANP molecule plays an important role in enhancing the affinity of antibodies.

4. GANP Gene Transferred Transgenic Non-Human Mammal

The target animal to be immunized with gp120 is a GANP gene transferred transgenic non-human mammal. Preferably, the GANP gene transferred transgenic non-human mammal is capable of expressing the transferred GANP gene in B cells.

(1) GANP Gene and its Related Molecules

Complexes formed by GANP gene and its related molecules are needed directly or indirectly in the process of induction of mutations in genes. When repairing genetic mutations, GANP protein has the ability to promote induction of mutations in the V region so

that high affinity antibodies are obtained. Therefore, the transgenic non-human mammal of the invention is capable of promoting the production of high affinity antibodies of acquired immunity because of the introduction of this GANP gene or a mutant thereof. Further, a transgenic non-human mammal overexpressing this gene is capable of promptly producing antibodies with high avidity to antigens. Therefore, by immunizing the above-described transgenic non-human mammal with a specific antigen, it is possible to obtain easily antibodies with high affinities which were unobtainable by conventional methods. As a result, it becomes possible to obtain polyclonal or monoclonal antibodies capable of eliminating obstinate pathogenic microorganisms or foreign substances. Further, by preparing humanized antibodies using the transgenic non-human mammal of the invention, or by preparing single chain antibodies comprising the V region of the antibody produced by the transgenic non-human mammal of the invention, it becomes possible to sharply increase the effect of antibody therapy.

Because of the GANP gene or its mutant transferred thereinto, the transgenic non-human mammal of the invention is capable of promoting the production of high affinity antibodies in B cells, and the high affinity antibody-producing cells have resistance to apoptosis induction signals.

(2) Mammals for Use in GANP Gene Transfer

The term "mammal" used in the present invention means any of non-human mammals such as bovine, horse, pig, goat, rabbit, dog, cat, mouse, rat, hamster and guinea pig. Preferably, mouse, rabbit, rat or hamster is used. Most preferably, mouse is used.

The transgenic non-human mammal of the invention may be prepared by introducing a GANP gene into unfertilized eggs, fertilized eggs, embryonic cells comprising spermatozoa and protocells thereof, preferably into cells of embryogenesis stage (more preferably, the single cell or fertilized egg cell stage and yet generally before eight-cell stage) in the development of non-human mammals, by a method such as the calcium phosphate method, electric pulsing, lipofection, aggregation, microinjection, the particle gun method, or the DEAE-dextran method. Further, it is also possible to transfer a GANP gene of interest into somatic cells, organs of the living body, tissue cells, etc. by the above-mentioned gene transfer methods to use the resultant cells, etc. for cell culture or tissue culture. Further, it is possible to create transgenic non-human mammals by fusing these cells with the above-described embryonic cells according to known cell fusion methods.

When a GANP gene is transferred into an animal of interest, it is preferred that the gene be transferred in the form of a gene construct in which the gene is ligated downstream

of a promoter capable of directing expression of this gene in cells of the animal of interest. Specifically, a vector in which a GANP gene is ligated downstream of various promoters capable of directing expression of the GANP gene derived from various mammals having the GANP gene of interest may be microinjected into fertilized eggs of the mammal of interest (e.g. mouse fertilized eggs) to thereby create a transgenic non-human mammal capable of high expression of the GANP gene of interest.

(3) Expression Vector

Examples of expression vectors for GANP gene include plasmids derived from *Escherichia coli*; plasmids derived from *Bacillus subtilis*; plasmids derived from yeast; bacteriophages such as λ-phage; retroviruses such as Moloney leukemia virus; and animal or insect viruses such as vaccinia virus or baculovirus.

As promoters for regulating gene expression, promoters of viruses-derived genes; promoters of various mammals (such as human, rabbit, dog, cat, guinea pig, hamster, rat and mouse)-derived genes; and promoters of birds (such as chicken)-derived genes may be used.

Examples of promoters of viruses-derived genes include promoters of cytomegalovirus-, Moloney leukemia virus-, JC virus-.or breast cancer virus-derived genes.

Examples of promoters of various mammals- and birds-derived genes include promoters of albumin, insulin II, erythropoietin, endothelin, osteocalcin, muscle creatine kinase, platelet-derived growth factor β, keratin K1, K10 and K14, collagen type I and type II, atrial natriuretic factor, dopamine β-hydroxylase, endothelial receptor tyrosine kinase, sodium/potassium-dependent adenosine triphosphatase, neurofilament light chain, metallothionein I and IIA, metalloproteinase I tissue inhibitor, MHC Class I antigen, smooth muscle α-actin, polypeptide chain elongation factor 1α (EF-1α), β-actin, α- and β-myosin heavy chains, myosin light chains 1 and 2, myelin basic protein, serum amyloid P component, myoglobin and renin genes.

The above-described vector may have a terminator which terminates the transcription of a messenger RNA of interest in a transgenic non-human mammal. For the purpose of achieving still higher expression of GANP gene, the splicing signal of each gene, enhancer region, or a part of an intron of an eukaryotic gene may be ligated upstream (5') of the promoter region, between the promoter region and the translation region, or downstream (3') of the translation region, if desired.

In a preferred embodiment of the invention, it is possible to allow selective expression of the transferred GANP gene in B cells by ligating the GANP gene downstream of an immunoglobulin promoter or by ligating a human immunoglobulin gene intron

enhancer moiety upstream (5') of the GANP gene.

(4) Transfer of GANP Gene

The transfer of GANP gene at the fertilized egg cell stage is preferably carried out in such a manner, for example, that excessive presence of GANP gene is secured in all the embryonic cells and somatic cells of the mammal of interest. Excessive presence of GANP gene in the embryo cells of the created animal after gene transfer means that all the progeny of that created animal has excessive GANP gene in all the embryonic cells and somatic cells. The progeny of this kind of animal which inherited the GANP gene has excessive GANP protein in all the embryonic cells and somatic cells.

In the present invention, first, heterozygotes which have the transferred gene in one of the homologous chromosomes are prepared; then, homozygotes which have the transferred gene in both of the homologous chromosomes are obtained by mating the heterozygotes with each other. Subsequently, by mating female homozygotes with male homozygotes, all the resultant progeny retains the transferred GANP gene stably. After confirmation of the excessive presence of GANP gene, the progeny may be bred in usual breeding environments.

Fertilized eggs of a non-human mammal of interest (preferably, mouse) or its ancestor (back-crossing) to be used for transferring a foreign GANP gene different from the endogenous gene of the mammal of interest are obtained by mating allogenic male and female mammals.

Although fertilized eggs may be obtained by natural mating, it is preferred that female mammals after artificial adjustment of their sexual cycle be mated with male mammals. As a method for artificially adjusting the sexual cycle of female mammals, such a method may be used preferably in which follicle-stimulating hormone (pregnant mare serum gonadotropin (PMSG)) and then luteinizing hormone (human chorionic gonadotropin (hCG)) are administered by, e.g., intraperitoneal injection.

After the transfer of a foreign GANP gene into the resultant fertilized eggs by the methods described above, the eggs are artificially transferred/implanted in female mammals. As a result, non-human mammals having a foreign gene-integrated DNA are obtained. In a preferable method, fertilized eggs are transferred/implanted artificially in pseudo-pregnant female mammals in which fertility has been induced by mating with male mammals after administration of luteinizing hormone-releasing hormone (LHRH). As totipotent cells into which a gene is to be transferred, fertilized eggs or early embryos may be used if the mammal of interest is mouse. As a method of gene transfer into cultured cells, DNA

microinjection is preferable in view of the production efficiency of transgenic non-human mammal individuals and the transmittance efficiency of the transgene to the subsequent generation.

Subsequently, the gene-injected fertilized eggs are transplanted into the oviduct of a recipient female mammal. Those animals which have developed from the eggs up to individuals and have been successively born are bred under foster parents. Then, DNA is extracted from a part of their bodies (e.g. the tail end in the case of mouse) and subjected to Southern analysis, PCR, etc. Thus, it is possible to confirm the presence of the transgene. Those animals in which the presence of the transgene has been confirmed are designated as founder animals. The transgene is transmitted to 50% of their offspring (F1). Further, by mating F1 individuals with wild-type animals or other F1 individuals, F2 individuals which have the transgene in one (heterozygote) or both (homozygote) of the diploid chromosomes can be produced.

Alternatively, transgenic non-human mammals expressing high levels of GANP protein may also be created by introducing the above-described GANP gene into ES (embryonic stem) cells. For example, the GANP gene is introduced into HPRT negative (i.e. lacking hypoxanthine-guanine phosphoribosyltransferase gene) ES cells derived from normal mouse blastocysts. Then, those ES cells in which the GANP gene has been integrated through homologous recombination induced in a mouse endogenous gene are selected by HAT selection. The thus selected ES cells are microinjected into fertilized eggs (blastocysts) obtained from other normal mouse. The resultant blastocysts are transferred into the uterus of other normal mouse as a recipient. Subsequently, chimeric transgenic mice are born from the recipient mouse. By mating these chimeric transgenic mice with normal mice, heterotransgenic mice can be obtained. Further, by mating the heterotransgenic mice with each other, homotransgenic mice can be obtained.

The present invention encompasses not only the above-described transgenic non-human mammal but also its progeny and a part of the transgenic non-human mammal or its progeny in the scope of the invention. As a part of the transgenic non-human mammal, a tissue, organ, cell or the like of the transgenic non-human mammal or its progeny may be enumerated. Specific examples of organs or tissues include the spleen, thymus, lymph nodes, bone marrow or tonsil; and specific examples of cells include B cells.

The transgenic non-human mammal of the invention may be mated with a mammal that further activates B cells. As a result of such mating, antibodies of still higher affinity can be produced.

Recently, it has been reported that when B cells are activated in peripheral lymph

nodes in MRL/lpr mouse, mutagenesis in the V region is further increased in the T cell region after B cells passed through the germinal center. The present inventors have also found that non-immunized MRL/lpr mouse shows high expression equivalent to the expression observed in *ganp* transgenic mouse which was created by ligating a GANP gene downstream of Ig promoter and enhancer. This suggests a possibility that, while high affinity antibodies are not produced against autoantigens normally, high affinity antibodies to autoantigens may be produced in this autoimmune disease mouse because of the abnormal activation of GANP molecule.

Then, still higher mutagenesis can be expected if autoimmune disease mouse such as MRL/lpr, NZB or (NZB x NZW)F1 is used as the above-mentioned animal that still activates B cells.

By creating a GANP transgenic mouse from MRL/lpr mouse utilizing what has been described above, it may be possible to create a super high affinity antibody-producing mouse. In other words, by mating the GANP gene overexpressing transgenic non-human mammal of the invention with various autoimmune disease model animals, it is possible to create mammals capable of producing high affinity antibodies.

5. Preparation of High Affinity Antibodies to HIV gp120

(1) High Affinity Antibodies

The term "antibody" used in the invention means the entire molecule of an antibody (either polyclonal or monoclonal) capable of binding to a peptide consisting of amino acids from positions 308 to 330 of the amino acid sequence of gp120 or a fragment of the above antibody. The isotype of the antibody of the invention is not particularly limited. The antibody of the invention may have any isotype, e.g. IgG (IgG_1, IgG_2, IgG_3, IgG_4), IgM, IgA (IgA_1, IgA_2), IgD or IgE.

In the present invention, an antibody having high reactivity with an antigen is called a high affinity antibody. The term "high affinity" used herein means that the ability of an antibody to bind to an antigen is high. In the present invention, a high affinity antibody refers to an antibody which has higher ability to bind to an antigen than those antibodies prepared using conventional animals such as mouse, and which is slow in dissociating from that antigen. This means that such an antibody is high and specific in the ability to bind to an epitope sterically and closely. Besides, the binding of such an antibody to the epitope induces changes not only in the epitope but also the structure of the antigen itself, to thereby show strong activities eventually (e.g. biological activities such as neutralization of toxicity, prevention of HIV infection, deactivation, and so forth).

The binding ability (i.e. affinity) of an antibody may be measured as dissociation constant (KD), dissociation rate constant (Kdiss) or association rate constant (Kass) by Scatchard analysis or with a surface plasmon resonance sensor called Biacore. Biacore systems in which three technologies of sensor chip, microflow system and SPR detection system are integrated are to measure the strength, rate and selectivity of molecular binding. This apparatus enables real time detection of biomolecules and monitoring of interactions among a plurality of molecules without using labels. Specific examples of Biacore systems include Biacore 3000, Biacore 2000, Biacore X, Biacore J and Biacore Q (all of them are manufactured by Biacore).

With the above-described Biacore system, parameters showing the affinity of antibodies, i.e. dissociation constant (KD), dissociation rate constant (Kdiss) [1/Sec] and association rate constant (Kass) [1/M.Sec] are measured.

Antibodies with smaller dissociation constant (KD) values are preferable because the smaller the dissociation constant value, the higher the affinity. The binding ability (affinity) of an antibody is determined by the two parameters of Kdiss and Kass, and is represented by the following formula:

KD (M) = Kdiss/Kass

Although the affinity of the resultant antibody varies depending on a plurality of factors such as types of antigen, KD value is preferably 1×10^{-9} (M) or less, more preferably 1.5×10^{-10} (M) or less, and still more preferably 1.0×10^{-10} (M) or less (especially, 9.9×10^{-11} (M) or less).

In the present invention, when the resultant antibody reveals any of the above-described effects or natures, the antibody is judged as a high affinity antibody.

The antibodies of the present invention (polyclonal antibodies and monoclonal antibodies and active fragments) may be prepared by any of various methods. Such antibody producing methods are well-known in the art.

(2) Preparation of Polyclonal Antibodies

The antigen prepared as described above is administered to a GANP transgenic non-human mammal. The kind of the mammal is not particularly limited. For example, rat, mouse or rabbit may be included. Among all, GANP transgenic mouse or GANP transgenic rabbit is preferable.

The dose of the antigen per animal is 5-50 mg when no adjuvant is used, and 0.5-2 mg when adjuvants are used. Examples of adjuvants include Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), aluminium hydroxide adjuvant, trehalose

dimycolate (TDM), lipopolysaccharide (LPS), silica adjuvant and commercial adjuvants. Immunization is performed by injecting the antigen intravenously, subcutaneously, and intraperitoneally, etc. The immunization interval is not particularly limited. Immunization is performed 1-10 times, preferably 2-3 times, at intervals of several days to several weeks, preferably 1-5 weeks. Six to sixty days after the final immunization, antibody titers are measured by such methods as ELISA (enzyme-linked immunosorbent assay), EIA (enzyme immunoassay) or RIA (radioimmuno assay). On the day when desired antibody titers are shown, blood is collected from animals to obtain antisera. When purification of the antibody is necessary in the above-described method of antibody collection, the antibody may be purified by appropriately selecting or combining known methods such as ammonium sulfate precipitation, ion exchange chromatography, gel filtration, affinity chromatography or the like.

Subsequently, the reactivities of polyclonal antibodies in the antisera are measured by ELISA or the like.

(3) Preparation of Monoclonal Antibodies

(a) Collection of Antibody-Producing Cells

The antigen prepared as described above is administered to a GANP transgenic non-human mammal (e.g., rat, mouse or rabbit). The dose of the antigen per animal is 0.05-2 mg when no adjuvant is used, and 0.05-2 mg when adjuvants are used. Examples of adjuvants include Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), aluminium hydroxide adjuvant, BCG, trehalose dimycolate (TDM), lipopolysaccharide (LPS) and silica adjuvant. Preferably, FCA and FIA are used in combination in view of the ability to induce antibodies, etc. Immunization is performed mainly by injecting the antigen intravenously, subcutaneously or intraperitoneally. After the first immunization, preferably, the animals are boosted several times; after passage of appropriate number of days, blood samples are taken and antibody titers are measured by the method described above. Since antibodies produced by the method of the invention are high affinity antibodies, the first immunization may be sufficient only with the first immunization. The immunization interval is not particularly limited. Immunization is performed 1-10 times, preferably 1-5 times, at intervals of several days to several weeks, preferably 2-5 weeks. One to sixty days, preferably 1-14 days, after the final immunization, antibody-producing cells are collected. As antibody-producing cells, splenocytes, lymph node cells, peripheral blood cells or the like may be enumerated. Among all, splenocytes or local lymph node cells are preferable.

The high affinity antibody-producing cells obtained as described above are also included in the present invention.

(b) Cell Fusion

When a GANP transgenic mouse is used, for example, cell fusion of antibody-producing cells and myeloma cells is conducted to obtain hybridoma cells. As a myeloma cell to be fused to the antibody-producing cell, a commonly available cell line derived from a mammal such as mouse may be used. Specifically, a preferable cell strain to be used in the invention has drug selectivity, cannot survive in HAT selection medium (containing hypoxanthine, aminopterin and thymidine) in unfused conditions, and can survive there only after fusion to antibody-producing cells. Specific examples of mouse myeloma cell strains useful in the invention include P3-X63.Ag8(X63), P3-X63.Ag8.U1(P3U1), P3/NS I/1-Ag4-1(NS1) and Sp2/0-Ag14(Sp2/0). In the selection of myeloma cells, compatibility with antibody-producing cells should be considered appropriately.

Subsequently, the above-described myeloma cell and the antibody-producing cell are fused. Briefly, $1x10^6$-$1x10^7$ cells/ml antibody-producing cells are mixed with $2x10^5$-$2x10^6$ cells/ml myeloma cells (preferable cell ratio of antibody-producing cells to myeloma cells is 5:1) in an animal cell culture medium such as serum-free DMEM or RPMI-1640, and fused in the presence of a cell fusion promoter. As the cell fusion promoter, polyethylene glycol with a mean molecular weight of 1000-6000 daltons (D) may be used. Alternatively, it is also possible to fuse antibody-producing cells and myeloma cells with a commercial cell fusion device utilizing electric stimulation (e.g., electroporation).

(c) Selection of Hybridomas and Cloning Thereof

Hybridomas of interest are selected from the cells after cell fusion. Briefly, the cell suspension is diluted, for example, with fetal bovine serum-containing RPMI-1640 medium and plated over microtiter plates. The selection medium is added to each well, and cells are cultured with appropriate exchange of the selection medium. Those cells which begin to grow about 14 days after the start of culture in the selection medium can be obtained as hybridomas.

Subsequently, screening is carried out to examine whether or not antibodies reactive with gp120 are present in the culture supernatant of the growing hybridomas. The screening of hybridomas may be performed by conventional methods and is not particularly limited. For example, aliquots of culture supernatants contained in those wells where hybridoma cells are growing are collected and subjected to screening by methods such as

ELISA, EIA or RIA.

The cloning of fused cells is performed by the limiting dilution culture method or the like. Those hybridomas producing an antibody showing strong reactivity with gp120 and having a KD value (indicator of affinity) of 1 x 10^9 (M) or less are selected and established.

(d) Collection of Monoclonal Antibodies

As a method of culturing the established hybridoma and collecting monoclonal antibodies from the resultant culture, a conventional cell culture method or the ascites formation method may be used. The term "culturing" means to grow the above-described hybridoma in a plate or dish, or to proliferate the above-described hybridoma in the abdominal cavity as described below. The term "culture" means any of the following: culture supernatant, cultured cells or disrupted cultured cells, or ascites.

In the cell culture method, the hybridoma cells are cultured in an animal cell culture medium (such as 10% fetal bovine serum-containing RPMI-1640 medium, MEM medium or serum-free medium) under conventional culture conditions (e.g., at 37°C under 5% CO_2) for 7 to 14 days. Then, the antibody of interest is obtained from the culture supernatant.

In the ascites formation method, the hybridoma cells are administered into the abdominal cavity of an allogenic animal (approx. $1x10^7$ cells/animal) to the mammal from which the myeloma cell used in the cell fusion was derived, to thereby expand the hybridoma cells greatly. One to two weeks thereafter, the ascites is collected.

When purification of antibodies is necessary in the above-described methods of monoclonal antibody collection, antibodies may be purified by a conventional method selected from ammonium sulfate precipitation, ion exchange chromatography, gel filtration, affinity chromatography or the like, or a combination of these methods.

(e) Use of the Binding Domain of Monoclonal Antibodies

Monoclonal antibodies bind to HIV antigen to thereby have activities to prevent HIV infection and neutralize and eliminate HIV. During this process, which V region gene is used in the H chain? Which D region gene or J region gene is used? Is N sequence inserted or not? Or which L chain V region gene is used? Is J region gene used or not? Information about these matters forms basis for creation of high affinity antibodies. However, binding affinity varies greatly not only by these matters but by the degree of somatic mutation of V region gene induced in peripheral lymphoid tissue. Here, the degree of somatic mutation is determined depending on the structures of regions involved in monoclonal antibody binding to antigen, i.e. the structures of each three CDR regions of the

H chain and the L chain. Therefore, with the use of information about the high affinity binding domain obtained by the present invention, it is possible to establish an anti-HIV antibody-producing cell from human EB virus-transformed memory B cell strain and introduce the information obtained here into the V region of the antibody by direct genetic engineering technique, to thereby obtain a high affinity antibody.

(4) Antibody Fragments, Humanized Antibodies or Human Antibodies

It should be noted that fragments of the above-described antibody and single chain antibodies of the V region of the above-described antibody are also within the scope of the present invention. A fragment of the antibody means a portion of the above-described polyclonal or monoclonal antibody. Specific examples of such a fragment include $F(ab')_2$, Fab', Fab, Fv (variable fragment of antibody), sFv, dsFv (disulphide stabilized Fv) or dAb (single domain antibody). A single chain antibody has a structure in which V_L (L chain variable region) and V_H (H chain variable region) are ligated with a linker.

The high affinity antibody of the invention may be a humanized antibody or human antibody. These human antibodies may be prepared by using mammals whose immune system has been replaced with the human immune system. After immunizing such mammals, human antibodies may be prepared directly in the same manner as used in the preparation of conventional monoclonal antibodies.

For the preparation of humanized antibodies, reconstructed variable regions consisting of human-derived framework regions (FR) and mouse-derived CDRs (complementarity determining regions) are prepared by transferring the CDRs of the variable regions in a mouse antibody into the human variable regions.

Subsequently, these humanized, reconstructed human variable regions are ligated to human constant regions. Methods for preparing humanized antibodies are well-known in the art.

Human antibodies may be produced using any animal in terms of structure, though generally the antigen binding site in the V region (i.e. hyper variable region) may raise some problem with respect to specificity and binding affinity. On the other hand, it is desirable that the structures of the remaining portion of the variable region and the constant region should be the same as the structures in human antibodies. With respect to genetic sequences common in human, genetic engineering techniques to prepare such sequences have been established.

(5) Characteristics of the Antibody

The antibody produced by the GANP transgenic non-human mammal of the invention has at least one of the following natures (i) to (iv).

(i) The antibody binds to the glycoprotein antigen gp120 with a molecular weight of 120 kD in the envelope of HIV and neutralizes HIV.

(ii) By binding to the surfaces of HIV-infected cells, the antibody inhibits syncytium formation induced by infected cells and non-infected T cells.

(iii) The antibody recognizes at least a part of region gp120(308-330) as epitope.

(iv) The antibody binds to at least a part of region gp120(308-330) with high affinity (KD = 1 x 10^9 (M) or less).

The syncytium formation means that an infected cell incorporates a non-infected cell into itself to thereby form one cell. When HIV is cultured with cells *in vitro*, sometimes syncytia are formed. Such syncytia cannot survive, and die. It is known that individuals infected with syncytium inducing (SI type) HIV show rapid decrease in $CD4^+$ lymphocytes and develop AIDS swiftly.

6. Pharmaceutical Compositions

The high affinity antibody of the invention which is raised against HIV (the causative of AIDS) as its antigen has an effect of neutralizing the activity of HIV. Therefore, the antibody of the invention is useful for therapeutic or prophylactic pharmaceutical compositions for AIDS. The pharmaceutical composition of the invention is provided comprising the high affinity antibody of the invention or a fragment thereof as an active ingredient, preferably provided in a form of a pharmaceutical composition comprising a pharmacologically acceptable carrier(s) additionally.

The "pharmacologically acceptable carrier" used herein includes excipients, diluents, fillers, disintegrants, stabilizers, preservatives, buffers, emulsifiers, aromatics, coloring agents, sweetening agents, thickening agents, flavoring agents, dissolution aids and other additives. By using one or more of these carriers, various forms of pharmaceutical compositions may be prepared, e.g. injections, solutions, capsules, suspensions, emulsions and syrups. These pharmaceutical compositions may be administered orally or parenterally. Other forms for parenteral administration include injections which comprise one or more active substances and are prescribed by conventional methods.

The dose of the drug of the invention varies depending on the age, sex, body weight and conditions of the patient, treatment effect, the method of administration, time period for treatment, or the type of the high affinity antibody (the active ingredient) contained in the drug. Usually, the drug of the invention may be administered to adults in the range from 10

μg to 1000 mg per administration, preferably in the range from 10 μg to 100 mg per administration. However, the dose is not limited to this range. When the body weight of a patient is 60 kg, the amount of his/her body fluid may be estimated 5 liters. In *in vitro* experiments, effective concentrations of antibodies are usually 5-50 μg/ml. According to simple calculation, it is desirable that 25-250 mg of the antibody is present in body for at least several days.

For example, in the case of injections, the antibody of the invention may be dissolved or suspended in a pharmacologically acceptable carrier (such as saline or commercial distilled water for injection) so that the antibody concentration is from 0.1 μg/ml (antibody/carrier) to 10 mg/ml (antibody/carrier). The thus prepared injection may be administered to human patients in need of treatment at a rate of 1 μg -100 mg/kg body weight, preferably at a rate of 50 μg -50 mg/kg body weight, per administration once to several times per day. The route of administration may be intravenous injection, subcutaneous injection, intradermal injection, intramuscular injection or intraperitoneal injection, for example. Among all, intravenous injection is preferable. Optionally, injections may be prepared in the form of a non-aqueous diluent (e.g. propylene glycol, polyethylene glycol, vegetable oil such as olive oil, alcohol such as ethanol), suspension or emulsion. Sterilization of such injections may be performed, for example, by filter-sterilization through a bacteria removal filter or addition of antiseptics. Injections may take a form that is prepared into an injection at the time of use. Briefly, a sterile solid composition is prepared by lyophilization or the like, and this solid composition may be dissolved in aseptic distilled water for injection or other solvent at the time of use.

7. HIV Detection Kit

The high affinity antibody of the invention is useful as a drug for diagnosing, treating or preventing diseases.

Detection of HIV infection using the antibody of the invention is carried out by binding samples taken from subjects (such as saliva or blood) and the antibody of the invention or a fragment thereof by antigen-antibody reaction, and determining the amount of antigen of interest in the sample from the amount of bound antibody. The amount of antibody may be detected by known methods of immunological measurement. For example, immunoprecipitation, immunoagglutination, labeled immunoassay, turbidity immunoassay or the like may be used. Labeled immunoassay is especially preferable because of simplicity and high sensitivity. In labeled immunoassay, the antibody titer in a sample is represented by the amount of label detected directly with a labeled antibody.

Alternatively, the antibody titer may be represented relatively using an antibody of known concentration or known titer as a standard solution. Briefly, a standard solution and a sample are measured with a meter; then, using the resultant value of the standard solution as a standard, the antibody titer in the sample may be expressed relatively. As a labeled immunoassay, any known method such as ELISA, EIA, RIA, FIA (fluoroimmunoassay) or luminescence immunoassay may be used.

By using the high affinity antibody of the invention, it is possible to evaluate the efficacy of AIDS therapeutics with high sensitivity. The method of efficacy evaluation using the high affinity antibody of the invention may be carried out as follows. Test drugs are administered to AIDS patients or AIDS model animals prepared by transplanting human lymphocytes (SCID-Hu mouse); then, the amounts of HIV in these bodies or the amounts of immuno deficient virus in model animal bodies are detected with the high affinity antibody of the invention. By comparing the resultant amounts, it is possible to evaluate the efficacies of test drugs as an AIDS therapeutic through the amounts of the antigen in bodies. At this time, the antibody of the invention is expected to have sensitivity 2- to 100-fold higher than that of conventional antibodies.

The high affinity antibody of the invention may be provided in a form of diagnosis kit for various diseases. This kit may be used in the diagnosis method and the efficacy evaluation method of the invention. Further, this kit may also be used as a highly sensitive, rapid and simple kit for checking the presence/absence of HIV infection in blood transfusion preparations and biological samples. The kit of the invention comprises at least one component selected from the following (a) and (b).

(a) The antibody of the invention or a labeled product thereof.

(b) An immobilizing reagent in which the antibody or labeled product of (a) above is fixed.

Here, the labeled product of antibody means an antibody labeled with an enzyme, radioactive isotope, fluorescent compound or chemiluminescent compound.

In addition to the above-described components, the kit of the invention may comprise other reagents to conduct the detection of the invention, e.g., when the labeled product is an enzyme-labeled antibody, an enzyme substrate (color developing substrate, etc.), enzyme substrate solution, enzyme reaction termination solution, or dilution for samples.

Hereinbelow, the present invention will be described in more detail with reference to the following Examples. However, the present invention is not limited by these

Examples.

In the following Example, three types of mice were used. They were Balb/c mouse (usually used in immunization), wild type (WT) mouse and GANP transgenic (Tg) mouse. Individuals of these three mice were immunized with as an immunizing antigen, HIV24NL43 (308-330) peptide (which is expected to neutralize HIV) linked to a carrier protein. Two individuals from each type of mouse were used to carry out cell fusion. The resultant cells were screened by ELISA and measurement with a Biacore system to thereby obtain positive hybridomas. Subsequently, purified antibodies obtained from individual hybridomas were analyzed by ELISA and measurement with a Biacore system.

The results revealed that monoclonal antibodies (3 clones) obtained from GANP transgenic (Tg) mouse are monoclonal antibodies with fairly high affinity. The dissociation constant (KD = k diss / k ass) value which is an indicator of affinity was 9.90×10^{-11}(M) in the clone with the highest affinity.

EXAMPLE 1: Preparation of GNAP Transgenic (Tg) Mouse

A transgene to be introduced into mice was prepared by inserting a 5.3 kb mouse GANP gene into the EcoRI site of pLG vector. This vector having a human immunoglobulin intron enhancer domain (2 kb EcoRI fragment) is a specific vector that directs strong expression in B cells. This gene was linearized and transferred into mice. Briefly, a linearized pLG vector (Koike, M. et al., Int. Immunol. 7, 21-30 (1995)) comprising the full-length mouse GANP cDNA was micro-injected into fertilized eggs of C57BL/6 mice. The presence of the transferred gene was screened using the genomic DNA obtained from mouse tail, the following primers and the reaction solution (upper panel, Fig. 1). In the upper panel of Fig. 1, the band appearing at around 5.3 kb represents the GANP gene.

1-5' primer: 5'-TCCCGCCTTCCAGCT GTGAC-3' (SEQ ID NO: 7)

1-3' primer: 5'-GTGCTGCTGTGTTATGTCCT-3' (SEQ ID NO: 8)

Composition of the reaction solution

DNA (50 ng/μl)	1 μl
10x buffer	2.0 μl
2.5 mM dNTP mix	2.0 μl
1-5' primer (10 μM)	0.8 μl
1-3' primer (10 μM)	0.8 μl
Z-Taq DNA polymerase	0.1 μl
dH_2O	13.3 μl

Reaction conditions:

[98°C 5 sec; 59°C 5 sec; 72°C 10 sec] x 35 cycles

4°C

Whether the expression of GANP mRNA is enhanced or not was examined by RT-PCR.

Total RNA was extracted from the spleen or splenic B cells using Trizol (Invitrogen). RT-PCR was performed with two primers (1-5' primer and 1-3' primer) to synthesize cDNA (Kuwahara, K. et al., Blood 95, 2321-2328 (2000)). GANP transcript was detected by agarose gel electrophoresis. β-actin transcript was used as a control.

As a result, GANP transgenic mice exhibited increased expression of GANP in B cells (lower panel, Fig. 1) and showed normal differentiation of B lineage cells in surface marker analysis of bone marrow, spleen and lymph node cells.

EXAMPLE 2: Preparation of Antibodies

(1) Materials

(a) Animals: Balb/c mouse, wild type (WT) mouse and GANP transgenic (Tg) mouse

(b) Immunizing antigen: HIV24NL43 peptide/KLH conjugation

(c) ELISA antigen: HIV24NL43 peptide (sequence: CNNTRKSIRI QRGPGRAFVT IGKI (SEQ ID NO: 9))

(d) Myeloma cell: P3-X63.Ag8.U1

(e) Secondary antibody: HRP-labeled anti-mouse antibodies IgG, IgA and IgM

(2) Methods

Five individuals each of the above-described three types of mice were immunized with NL43 peptide (carrier protein: KLH) three times at intervals of two weeks. After the third immunization, antisera were collected and subjected to ELISA to measure antibody titers.

Two individuals showing high antibody titers were selected from each type of mice used, and antibody-producing cells (splenic cells) were collected therefrom and fused to P3U1 myeloma cell. Cells were plated to give a density of 0.2×10^5 GANP-Tg mouse splenic cells/well. Balb/c mouse-derived 5448 clones, wild-type (WT) mouse-derived 1888 clones and GANP transgenic (Tg) mouse-derived 2016 clones were cultured in HAT medium.

After day 9 of HAT culture, culture supernatants were collected and subjected to ELISA using NL43 peptide (1 μg/ml) as an immobilized antigen. From the individual

populations of culture supernatants from GANP-Tg mice and WT mice, those clones showing an absorbance at 490 nm of 1.50 or more as determined by ELISA were selected and cloned in HT medium.

After day 9 of HT culture, culture supernatants were collected and subjected to ELISA using NL43 peptide (1 μg/ml) as an immobilized antigen. As a result, three hybridoma clones were established from Balb/c mice (B1-10, B2-24 and B2-27), 9 hybridoma clones were established from WT mice (W1-2, W1-7, W1-8, W1-10, W1-21, W1-43, W1-45, W1-63 and W1-84) and 8 hybridoma clones were established from GANP Tg mice (G1-22, G1-68, G1-124, G1-165, G1-181, G2-231, G2-10 and G2-25).

Of these clones, G2-25 was designated "Anti-NL43mono. Clone No. G2-25 hybridoma cell" and internationally deposited at International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology (Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan; zip code No. 305-8566) on February 25, 2004 under the accession number of FERM BP-08644 according to the Budapest Treaty.

Individual clones from GANP-Tg mice and WT mice were cultured in RPMI medium, and then further cultured in a serum free medium SFM. These clones were purified with protein G to thereby obtain anti-peptide monoclonal, purified antibodies.

EXAMPLE 3: Measurement of Affinity

Using the monoclonal antibodies prepared in Example 2 above, the following evaluation and examination were carried out.

In order to evaluate the affinity of each antibody, analyses by ELISA and with a Biacore system were conducted.

First, in ELISA, HIV24NL43 peptide (1 μg/ml) was used as immobilized antigen and immobilized at room temperature for one hour. The antigen-immobilized plate was washed with PBSTween 20 and blocked with 2.0% skim milk. After further washing with PBSTween 20, the antigen was reacted with the anti-peptide monoclonal antibodies (0.457-1 μg/ml) to be evaluated, at room temperature for one hour. Then, the resultant samples were washed with PBSTween 20 and reacted with HRP-labeled anti-mouse IgG, IgA or IgM at room temperature for one hour. After washing with PBSTween 20, color was developed with ortho-phenylene diamine (OPD) for 5 minutes, followed by termination of the reaction with 2N sulfuric acid.

Absorbance was measured with an ELISA plate reader at 490 mm.

The results of ELISA are shown in Fig. 2.

By using GANP-Tg mice, three antibodies with extremely high binding ability

were produced (Fig. 2, absorbance around 1.4-2.1). These monoclonal antibody clones are G1-181, G2-10 and G2-25 according to descending order of absorbance.

Subsequently, the physicochemical binding ability of each antibody was examined with a Biacore system.

Briefly, HIV24NL43 peptide was bound to a Biacore sensor chip as a ligand. As analyte solutions, solutions of the anti-peptide monoclonal antibodies were used. Association rate constant (k ass), dissociation rate constant (k diss) and dissociation constant KD (KD=k diss/k ass) that is an indicator of affinity were calculated for each of the antibodies. The smaller the KD value is, the higher the affinity is evaluated.

As a comprehensive evaluation of the affinity of antibodies, the dissociation constant values and the results of ELISA for individual clones are shown in Fig. 3. In Fig. 3, relations between clones and dissociation constant values are as shown below.

Clone	Dissociation Constant (M)
G1-181	1.09×10^{-8}
G2-10	9.90×10^{-11}
G2-25	1.51×10^{-10}

At present, measurement of sensitivity with a Biacore system is most effective for examining the binding affinity of antibodies. In this method, dissociation constant is conveniently calculated as a numerical value obtained by dividing the association rate constant of an antibody binding in a unit time by the dissociation constant of the bound antibody. With respect to the activity of antibodies, how quickly antibodies bind to the antigen is also an important factor in addition to the affinity to the antigen peptide. Antibodies are expected to bind to the virus in the living body as swiftly as possible, alter the molecular structure of the viral antigen and enter a still more firm state of binding. Although the binding affinity of clone G1-181 is not so high in view of the calculated dissociation constant value, this clone is excellent in the profile of association constant that this clone binds to a great number of antigen molecules most rapidly.

Usually, antibodies obtained from Balb/c mouse which is commonly used for monoclonal antibody preparation have a dissociation constant (KD) value of 4.97×10^{-6} to 5.68×10^{-9} (M), and thus are low affinity antibodies. Besides, only a small number of antibodies can be obtained. In wild type (WT) mouse which is a negative control, the dissociation constant (KD) value remained in a range from 2.81×10^{-5} to 3.11×10^{-9} (M); thus, binding affinity was limited.

On the other hand, it is possible to obtain a high affinity antibody (G2-10) with a dissociation constant (KD) value of 9.90×10^{-11} (M) from GANP transgenic (Tg) mouse. The affinity of this antibody can be 57 times higher than that of Balb/c mouse clones and 31 times higher than that of wild type (WT) mouse clones.

EXAMPLE 4: Binding of the Monoclonal Antibodies to NL43 Envelope

A binding assay was performed to examine whether anti-HIV peptide (NL43) monoclonal antibodies prepared in Example 2 above have the binding ability to actual NL43 (HIV's envelope protein) *in vitro*.

(1) Materials

(a) Anti-HIV (NL43) purified antibodies

The antibodies prepared in Example 2 were used. Specifically, the following antibodies were used.

Balb/c mouse: 3 clones (B1-10, B2-24 and B2-27)

Wild type (WT) mouse: 9 clones (W1-2, W1-7, W1-8, W1-10, W1-21, W1-43, W1-45, W1-63 and W1-84)

GANP transgenic (Tg) mouse: 8 clones (G1-22, G1-68, G1-124, G1-165, G1-181, G1-231, G2-10 and G2-25)

As controls, 70Z/3 2-28, 0.5β and anti-CD19 were used.

(b) Plasmid Vectors

pLP-IRES2-EGFP (Clontech) and pLP-NL4-3 envelope-EGFP were used. By using these vectors, it becomes possible to translate both the gene of interest (NL43) and EGFP from a single RNA. Thus, almost 100% of the fluorescence-emitting cells express NL43.

(c) Gene Transfer Reagent

Effectene Transfection Reagent (QIAGEN) was used.

(d) Secondary Antibody

APC-labeled goat anti-mouse IgG antibody (BD Pharmingen) was used.

(2) Methods

In the binding assay, GFP is introduced into a human embryonic renal cancer cell line (293T cells) for monitoring the expression of envelope (NL43). The resultant cells are reacted with anti-HIV (NL43) purified antibodies and APC-labeled secondary antibody to thereby stain cell surfaces. Subsequently, two-color analysis is conducted by flow cytometry. Then, the binding ability to the envelope is evaluated with fluorescence intensity.

GFP gene was introduced into cells as described below. 400000 cells of human embryonic renal cancer cell line (293T cells) were plated in a 10 cm dish and cultured for one day. Then, pLP-IRES2-EGFP (Clontech) or pLP-NL4-3-EGFP (5 μg each) was introduced to the cells using Effectene Transfection Reagent. After a 36 hour culture, cells were collected and cell surfaces were stained. The staining of cell surfaces was performed using 10 μg/ml each anti-HIV (NL43) purified antibody and a 50-fold diluted APC-labeled goat anti-mouse IgG antibody. The cells were reacted with the anti-HIV antibody and the APC-labeled antibody for 30 minutes each on ice.

The binding ability of each anti-HIV monoclonal antibody to the envelope was evaluated by calculating mean fluorescence intensity (MFI) using FACS Calibur.

(3) Results

The results of the binding assay are shown in Figs. 4 and 5. Figs. 4 and 5 are bar graphs showing mean fluorescence intensity (MFI) when GFP positive (+) and GFP negative (-) cells were gated, respectively. These bar graphs show that in pLP-NL4-3 envelope-EGFP-introduced cells antibodies bind to the envelope more effectively when APC mean fluorescent intensity (MFI) in GFP positive cells is higher. The results of the binding assay revealed that monoclonal antibodies produced in GANP transgenic (Tg) mice (e.g., G1-22, G1-68, G2-10 and G2-25 clones) have the ability to bind to envelope.

EXAMPLE 5: Neutralizing Activities of Monoclonal Antibodies

In order to examine whether the purified monoclonal antibodies used in Example 4 actually have the ability to inhibit HIV-1 infection, a neutralizing activity experiment (viral infection inhibitory experiment) was conducted in human CD4 positive cells using the monoclonal antibodies.

(1) Materials

(a) Anti-HIV (NL43) purified antibodies

Each of the anti-HIV (NL43) purified antibodies used in Example 4 was used. As controls, 70Z/3 2-28 and 0.5β were used.

(b) HIV-1 Stock Strain

PM1 cells grown in 10% inactivated fetal bovine serum-added RPMI-1640 medium and stored at -80°C were used.

(c) β Galactosidase detection kit

Galacto-star (TROPIX) was used as a β galactosidase detection kit. In AIDS virus infection inhibitory (neutralizing activity) experiments, this kit detects the β galactosidase produced by CD4 cells (MAGI/CCR5) with a chemiluminescence substrate (the

Reed-Muench method) to thereby judge the death or survival of CD4 cells (viable cell count).

(2) Methods

When a specific amount of HIV is added to CD4 cells (MAGI/CCR5) after one day culture, β galactosidase becomes undetectable from AIDS virus-infected cells. In this system, the neutralizing activities of antibodies are measured as follows. Immediately before the addition of AIDS virus, anti-HIV (NL43) purified antibodies different in efficacy are added to the above-described MAGI/CCR5 cells in advance. Then, whether or not these antibodies can inhibit viral infection when HIV is added is evaluated using the yield of β galactosidase as an indicator.

This infection measuring system enables to determine with high sensitivity the amount of virus necessary for viral infection. As a result of pilot examination on MAGI/CCR5 cells, the amount of virus to be added was determined 500 based on the 50% endpoint tissue culture infectious dose (TCID50) of the virus.

In order to infect cells with HIV, MAGI/CCR5 cells were cultured in 96-well plates at a density of 1 x 10^4 cells/well. After one day, 50 μl of each antibody was added and incubated at 37°C for 30 minutes. Subsequently, 50 μl of HIV-1 solution reacted with 10 μg/ml DEAE-dextran was added and incubated. Each of the antibodies was added at three different concentrations: 0.5, 5 and 50 μg/ml. After two days, β galactosidase activities were measured with Galacto-star (TROPIX).

(3) Results

The results of the neutralizing activity experiment are shown in Figs. 6 and 7. The results of measurement of the neutralizing activities of individual antibodies revealed that G2-10 and G2-25 clones produced by GANP transgenic (Tg) mice have the HIV neutralizing activities as shown in Table 1 at indicated concentrations.

Table 1

Clone	Concentration	Neutralizing Activity (Infection Inhibitory Ability)
G2-10	50 μg/ml	98.2%±1.2
	5 μg/ml	93.5%±1.7
	0.5 μg/ml	70.9%±9.7
G2-25	50 μg/ml	101.2%±0.4
	5 μg/ml	97.6%±1.6
	0.5 μg/ml	86.3%±2.4
0.5β	0.5 μg/ml	86.5%±3.0

When the antibody is actually used as a therapeutic antibody, it is important for the antibody to have the ability to inhibit viral infection at an extremely low dose. From this viewpoint, the values achieved by G2-10 and G2-25 show that these antibodies manifest HIV infection inhibitory ability at extremely low concentrations and these antibodies may be considered to be used as effective HIV therapeutics.

As a positive control in the measurement of neutralizing activities, an antibody (0.5β) which is said to have been prepared by immunizing with HIV envelope itself (Japanese Patent No. 2797099) was used. The neutralizing activity of 0.5β at 0.5 μg/ml was as high as 86.5%±3.0. However, the *in vitro* infection inhibitory ability in the above two clones (in particular, G2-25) can be said equivalent or superior to that ability of this antibody (0.5β).

In contrast, the 9 antibody clones produced by wild type (WT) mice showed almost no neutralizing activity at a low concentration of 0.5 μg/ml.

The results obtained in Examples 4 and 5 show that the anti-AIDS peptide (NL43) monoclonal antibodies produced in GANP transgenic (Tg) mice actually bind to HIV-1 viral envelope *in vitro* (Example 4) and that this binding has a strong neutralizing activity (infection inhibitory effect) (Example 5). More importantly, since these monoclonal antibody show a dissociation constant (KD) value of 9.90×10^{-11}, they are a group of high affinity antibodies which are capable of binding to the virus for a long period once they have bound thereto (Example 3).

High affinity monoclonal antibodies that have such a strong virus neutralizing effect and have remarkable low rate of dissociating with antigen are excellent antibodies which cannot be prepared by the conventional monoclonal antibody preparation attempted by a great number of laboratories based on a peptide sequence deduced from the genetic

sequence of HIV-1 virus. While those monoclonal antibodies prepared by immunizing wild type C57BL/6 mice show a high neutralizing activity only at a concentration of 50 μg/ml, both of GANP transgenic (Tg) mice-derived G2-10 and G2-25 monoclonal antibodies manifest a neutralizing activity equivalent to thereto at an extremely low concentration of 0.5 μg/ml. According to simple calculation, the latter antibodies show 100-fold higher neutralizing activity per protein concentration, and the binding of the latter antibodies is expected to last for a longer period by the order of 10^2.

These mouse anti-HIV-1 monoclonal antibodies have the following advantages: (a) they are high affinity antibodies specific to a viral peptide sequence; (b) they actually bind to HIV-1 envelope *in vitro*; (c) they are capable of inhibiting HIV-1 infection of human CD4 positive cells *in vitro*; (d) they may be antibodies to a novel epitope because they are produced by a mutant mouse not used in conventional methods; (e) this new VH region provides basic information for preparing still powerful antibodies by adding further genetic alteration by biotechnology.

The fact that it is possible to obtain swiftly high affinity antibodies having AIDS virus infection inhibitory effect using GANP transgenic (Tg) mice proves that the results of researches so far made and the plan of application thereof are reasonable. Therefore, the present invention is extremely useful as an invention which leads to development of an epoch-making therapy in the modern world threatened by infectious disease.

SEQUENCE LISTING FREE TEXT

SEQ ID NO: 7: primer

SEQ ID NO: 8: primer

INDUSTRIAL APPLICABILITY

According to the present invention, a high affinity anti-HIV antibody obtainable from a GANP gene transgenic non-human mammal and a pharmaceutical composition comprising the antibody are provided. Since the antibody of the invention has high affinity represented by its dissociation constant (KD) value of 1.0×10^{-9} (M) or less, a pharmaceutical composition comprising the antibody of the invention may be used as a therapeutic for acquired immunodeficiency syndrome (AIDS).

Further, according to the present invention, a cell producing the antibody and an HIV detection kit using the antibody are also provided.